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Inorganic Mercury Uptake and Mercury Tolerance Demonstrated by Several Species of Algae

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INORGANIC MERCURY UPTAKE AND MERCURY TOLERANCE DEMONSTRATED BY SEVERAL SPECIES OF ALGAE

by

Daniel S. Filip

A thesis submitted in partial fulfillment
of the requirements for the degree

of

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Committee Member

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Daniel S. Filip

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ABSTRACT

Inorganic Mercury Uptake and Mercury Tolerance

Demonstrated by Several Species of Algae

by

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Utah State University, 1972

Major Professor: Dr. Raymond I. Lynn
Department: Botany

The inhibition by low concentrations of inorganic mercury has been established for several species of freshwater algae, and a method has been developed to determine mercury levels in algal cells, having good precision at levels as low as one hundred parts per billion.

Rapid sorption of inorganic mercury by glassware was minimized by using heavy algal cultures and short term incubations. The rates and extent of mercury uptake by three species of freshwater green algae were delineated under laboratory conditions. Replicate mercury containing cultures yielded slightly different mercury concentrations upon analysis due to analytical errors and glass sorption. Therefore, percent mercury uptake by cells of available mercury at the time of sampling was found to be a reliable index for the rate, extent, and mechanism of mercury uptake.

(61 pages)

INTRODUCTION

Mercury contamination of natural water systems is now a recognized environmental hazard (Nelson, 1971; Wallace et al., 1971; Grant, 1971). The disposition of mercury in the environment continued unchecked until recently when accumulations were found to be potentially dangerous to man. Since outbreaks of mercury poisoning among people were first positively diagnosed in the early 1960's, Japan, Canada, Sweden, and the United States have actively engaged in research and established regulations to prevent further human poisoning (Council on Environmental Quality, 1971). Much research has been devoted to the accumulation of mercury in fish and aquatic birds which may be consumed directly by man. Yet relatively little work has been directed at mercury uptake by lower aquatic organisms.

At the base of the aquatic food web are the algae from which the majority of aquatic organisms derive most of their energy. It is therefore important to explore the pathways by which animals may take in and accumulate high concentrations of mercury from organisms at lower trophic levels. The present study focuses on the toxicity of mercury to several representative species of freshwater algae. The uptake of inorganic mercuric chloride by algae in laboratory cultures and the mechanism of mercury sorption has received foremost attention in this study.

REVIEW OF LITERATURE

Sources of environmental contamination

The major sources of mercury contamination of aquatic systems are industrial effluents, principally from chloralkali factories and pulp and paper industries. According to the U.S. Geological Survey (1970), industrial losses of mercury in the U.S. alone are recorded at 600 tons per year with mercury concentration in these effluents reaching 100,000 parts per billion (hereafter referred to as ppb). Various mercury compounds are used as catalysts in the production of acetaldehyde and vinyl chloride which are used extensively in chemical syntheses. Mercurials employed as slimicides were used extensively in paper production. A U.S. Food and Drug Administration ruling concerning paper food packaging forced the industry to use less toxic slimicides, and since the early 1960's this source of contamination has sharply diminished (Wallace et al., 1971).

Mining and smelting of mercury results in the output of the element in vapor form which readily diffuses through the air and water. Likewise, mercury tends to be associated with other metal ores and much is released in smelting operations for zinc, tin, gold, and copper (Wallace et al., 1971). Fossil fuels may sometimes contain mercury in heavy concentrations. In the United States, anthracite coal contains 1200 to 2700 ppm mercury, and crude oil up to 21,000 ppb (U.S. Geological Survey, 1970). Nelson (1971) estimated that in the combustion of coal in the U.S., 100 million pounds of mercury is released each year.

Chemistry of mercury and mode of toxicity

Mercury which is released in sewage or industrial effluents may enter the environment in many different states, some more toxic than others. Mercury exists in five positive valence states besides its metallic form: 2, 3, 4, 6, and 7. This variation enables it to complex with many other elements forming numerous inorganic and organic compounds. Methylmercury is reputed to be the most toxic and persistent chemical species, yet phenyl and ethylmercury are also potent forms (Grant, 1971).

Although only 2 percent of the inorganic mercury ingested is absorbed by mammals, 90-95 percent of methylmercury is absorbed by mammalian intestines (Grant, 1971). Methylation of inorganic mercury by bacteria was demonstrated in the avian intestine and may also occur in mammalian viscera. Once methylmercury is absorbed, it is transported through the mammalian blood stream by red blood cells (Grant, 1971). Though the mechanism of tissue specificity is unknown, much of the mercury accumulates in the kidney, liver, and cranial tissue (Wallace et al., 1971).

The toxicity of mercury to all organisms is caused by its tendency to bind with sulfhydryl groups of hydrolases and other enzymes, thereby inactivating these enzymes. In the kidney and most other tissues of birds, fish, and mammals, the poisoning effect lasts until mercury is effectively removed by excretion, yet because brain cells are not regenerated, damage to the brain is permanent (Wallace et al., 1971). Methylmercury has a high affinity for cranial tissues but inorganic mercury reaches the brain only when it is in its vapor

state (Grant, 1971). Within the dividing cell mercury interrupts mitotic spindle formation, causing non-disjunction of chromosomes resulting in birth defects in birds and mammals (Grant, 1971). Also, methylmercury can pass through the placental barrier in mammals causing congenital mental retardation. Metallic mercury, inorganic mercury, and aryl compounds are likewise detrimental but are generally less toxic and persistent than methylmercury (Nelson, 1971).

Transformations of mercury

Grant (1971) noted that 95-100 percent of mercury found in fish in methylmercury. Methylmercury is quite volatile and diffuses readily in aqueous systems, and this property may contribute to the long-lived nature of mercury. Yet, much of the mercury contamination is in forms other than the methyl state. Physical conditions of the water as well as biological constituents play an important role in dictating the chemical status of mercury. Under reducing conditions as in anaerobic lake bottom sediments, mercuric sulfide predominates. In an oxidizing environment the sulfide is gradually converted to sulfate (Wallace et al., 1971). Also, aerobically formed metallic mercury may volatilize in sunlight or be transformed biologically into an organic complex (Wallace, et al., 1971).

Jenson and Jernelov (1969) demonstrated conclusively that microorganisms in lake bottom sediments can methylate mercury. Fimreite (1970) noted that members of the genus Methanobacterium particularly are able to methylate metallic and inorganic mercury under anaerobic conditions, but the process occurs more efficiently aerobically (Nelson, 1971). Grant (1971) observed that

fish not only ingest mercury from lower trophic levels but also respire merthylmercury directly through their gills. He also pointed to the hypothesis that since fish accumulate merthylmercury at a high rate even when the water contains inorganic mercury, the gills of fish may contain methanogenic bacteria. In extracellular studies, Imura et al. (1971) found that an analog of Vitamin B_{12}, methylcobalamin, chemically methylates inorganic mercury. Since methylcobalamin has been isolated from bacteria and even mammalian cells, these findings offer a possible mechanism for natural methylation. Matsumura, Gotoh, and Boush (1971) showed that salts of phenylmercury compounds which are used as fungicides and slimicides cannot directly be transformed to methylmercury as previously believed. Their postulation that microorganisms transform mercury to the least toxic state possible is thus of particular interest. Whereas certain microorganisms methylate mercury, other bacteria change organomercury complexes into metallic mercury, a procedure which makes transformations seem cyclic (Wallace et al., 1971).

Because of the high affinity of mercury for sulfur-containing organic matter, the variation of mercury content in stream sediments can seemingly be explained by the chemical nature of the sediments (Feick, Horne, and Yeaple, 1972). Since the chloride ion complexes very strongly with the mercuric (Hg^{+2}) ion, high concentrations of calcium chloride and sodium chloride may make mercury more available in aquatic systems. Addition of these salts to water with mercury in the sediments resulted in a relative increase of 200-500 percent of detectable mercury in the water. Increasing the salt concentration

altered the pH of the water which also contributed to the release of mercury (Feick et al., 1972). Such findings would indicate a mechanism by which any mercury in sediment-rich fresh water would be released readily in estuarine environments where fresh water and salt water are mixed (Feick et al., 1972). Because mercury is readily transformed, all compounds of mercury released into the environment are potentially lethal.

Toxicity of mercury to various organisms

In freshwater algae, 1.0 ppb methylmercury or phenylmercury reduced photosynthesis by 40-50 percent of controls (Harris, White, and Macfarlane, 1970). Horowitz (1957) reported that vapor from 1.5 grams of metallic mercury in the sidearm of a Warburg vessel was sufficient to decrease photosynthesis significantly in Scenedesmus obliquus and at the same time increase respiration 300 percent. Matson, Mustoe, and Chang (1972) demonstrated that 3.5 ppm mercury as mercuric chloride inhibited galactolipid biosynthesis 50 percent and retarded chlorophyll synthesis 98 percent in the freshwater algae Ankistrodesmus braunii. The same concentrations of mercuric chloride accounted for a slightly lesser inhibition of Euglena gracilis biosynthesis activities. Methyl mercuric chloride in concentrations of 2.0 ppm caused an 85 percent reduction in galactolipid production and inhibited chlorophyll synthesis 98 percent in A. braunii. Both mercury compounds strongly inhibited galactosyl transferase activity by Euglena chloroplasts (Matson et al., 1972).

Daphnia magna, a zooplanktonic primary consumer which grazes on algae, was found to be very sensitive to mercury poisoning, tolerating only 0.6 ppb mercury as mercuric chloride (Anderson, 1948).

Goldfish exposed to a concentration of 820 ppb mercuric chloride died within 7 days, and an exposure for only 2 days to 3 ppb produced a significant impairment in learning behavior (Wallace et al., 1971). Such sublethal, chronic effects are important to species in an aquatic environment since such afflictions could interrupt the organism's ability to feed or avoid predators (Wallace et al., 1971).

Accumulation at various trophic levels

One of the most important problems concerning mercury contamination of aquatic systems is the accumulation in certain organisms and subsequent passage through the food web. Limited information concerning the accumulation of mercury in the primary aquatic trophic level is available. Terrestial vascular plants, which may directly or indirectly contribute to the aquatic web, accumulated little or no mercury in edible plant parts when they were grown in soil containing inorganic mercury (Wallace et al., 1971). Accumulation of mercury in a marine diatom was shown to occur primarily by passive surface sorption (Glooschenko, 1969).

There is some quantitative evidence of mercury accumulation by zooplankton and other primary consumers demonstrated by a comparison of mercury content in organisms in a stream above and below a paper mill. Wallace et al. (1971) reported that the Isopod (Asellus sp.) contained in its body 0.06 ppm mercury 15 km above the mill and 1.90 ppm 20 km below the mill. The Caddis-fly (Trichoptera sp.) larvae had 0.05 ppm above the mill but had accumulated 17.00 ppm 1 km downstream from the mill. Leeches (Hellobdella

sp.) tested contained 0.02 ppm above the mill and 4.40 ppm below the mill. Stone fly (Plecoptera sp.) and alder fly (Sialis sp.) larvae also accumulated mercury from the paper plant effluent. Regardless of the mechanism of accumulation in lower aquatic organisms, mercury is ultimately passed to animals at higher trophic levels such as fish (Grant, 1971).

In a study on precisely aged lake trout (Salvelinus namaycush) in Lake Cayuga, Ithaca, New York, Bache, Butenman, and Lisk (1971) found a positive correlation between the age of fish and the concentration of mercury in their tissues. Year old fish had an average accumulation of 0.24 ppm mercury; 3 year old fish had 0.38 ppm; 8 year old fish 0.55 ppm; and 12 year old fish 0.59 ppm. Also noted was an increase in percentage of total mercury as methylmercury, from 32 percent methylmercury in the yearling fish to an average of 78 percent methylmercury in 12 year old specimens. The primary sources of contamination were apparently coal burning power plants in the area. Fimreite (1970) has shown a correlation between body weight and mercury content in a variety of fish species taken from Canadian lakes and rivers where mercury contamination was suspected. A notably high concentration, 5.78 ppm, was recorded in lake trout (Salvelinus namaycush) from Pinchi Lake, British Columbia. Yellow walleye (Stizostedion sp.) from Lake Huron and Lake St. Clair averaged 1.08 and 2.88 ppm respectively. From the St. Clair River, rock bass (Ambloplites rupestris) registered 2.30 ppm, sunfish (Lepomia sp.) had 2.64 ppm, and white bass (Roccus chrysops) contained 1.62 ppm mercury.

From the same areas Fimreite also analyzed fish-eating birds. From Baie de Chaleur, New Brunswick, the livers of an immature and an adult great

blue heron (Ardea herodias) contained 4.53 and 11.30 ppm mercury respectively, the livers of four common terns (Sterna hirundo) averaged 2.50 ppm, and eggs from mergansers (Mergus sp.) registered 0 8 ppm. From Pinchi Lake the livers of three red-necked grebes (Podiceps grisegena) averaged 10.32 ppm mercury. In the case of Pinchi Lake, a mercury mine was in close proximity. In 1968, mining operations were reopened after 25 years. However, since specimens taken prior to 1968 also contained abnormally high concentrations of mercury, the hypothesis was supported that unless mercury is made biologically unavailable its effects in aquatic systems may last for decades (Fimreite, 1970).

Jernelov (1968), reporting findings from the analysis of feathers taken from specimens of aquatic predatory birds, found that since 1890 mercury concentrations have continued to rise. A proportional increase in mercury levels in several species of fish preyed on by these birds was noted (Miller et al., 1972).

The significance of some reports of mercury contamination may be overstated. Extensive marine contamination was thought to have occurred when swordfish and tuna were found to have mercury concentrations well in excess of the 0.5 ppm maximum permissible level for mercury in commercially sold fish established by the U.S. Food and Drug Administration (Miller et al., 1972). As a result of such findings large-scale confiscations of tuna and swordfish from many markets followed, resulting in economic losses of millions of dollars (Council on Environmental Quality, 1971). Recent data shows high levels of mercury in canned and freshly caught tuna and swordfish, but the analyses also

show conclusively that museum specimens of fish caught in 1878 assay as much mercury as present day specimens (Miller et al., 1972). However, fish and shellfish from marine and estuarine environments have accumulated high levels of mercury where there has been a direct influx of mercury (Jernelov, 1968). Methylmercury concentrations of 20-40 ppm were found in shellfish and fish taken from Minamata Bay, Japan, where 43 people died of mercury poisoning and many others suffered permanent brain damage during the 1950's. The source of the contamination was a factory producing acetaldehyde (Jernelov, 1968). Although mercury contamination of some marine fish may have been overstated in the case of tuna and swordfish, there is considerable evidence that mercury contamination in freshwater systems has definitely increased mercury levels in some freshwater fish (Miller et al., 1972).

There are many gaps in the knowledge of the full scope of mercury contamination problems. The literature is sometimes contradictory, particularly concerning levels of toxicity, avenues of accumulation, and mechanisms of mercury transformations. Such inconsistencies and gaps may be partially remedied by more intensive studies focusing on the toxicity and accumulation of mercury in species selected from all trophic levels.

METHODS OF PROCEDURE

Preparation of media

Precautions were taken against contamination. Bristol's medium (Starr, 1964; see Appendix for media preparation) was used in all phases of experimentation because, being a completely inorganic medium, it does not encourage bacterial growth. Since fungal contamination was also possible, the media was autoclaved after preparation. In control experiments, Bristol's was found to be free of detectable levels of mercury. All glassware was rinsed prior to use with concentrated nitric acid to prevent mercury contamination.

Toxicity of mercury to algae

To determine the tolerance of various species of algae to mercury, serial dilutions of a concentrated mercury stock (10^4 ppm mercury as mercuric chloride) were prepared in mercury-free Bristol's medium. Ten ml of media with known concentrations of mercury was transferred into optically matched 18 x 150 mm test tubes. One drop (about 0.05 ml) of exponentially growing, mercury-free algal stock culture was introduced to culture tubes. Although test tube cultures proved unsatisfactory for accurate tolerance and uptake analyses due to rapid sorption of the mercury by the glass, the cultures did demonstrate nonlethal, workable concentrations of mercury for the three species of algae chosen for investigation (Selenastrum capricornutum, Chlamydomonas reinhardi, and Chlorella pyrenoidosa). All were grown aseptically from stock

cultures obtained from the Indiana University Algal Collection, Bloomington, Indiana. In all three species, 0.1 ppm mercury proved only slightly inhibitory and was chosen as the concentration to be used in all uptake studies.

Lethal and inhibitory levels of mercury were best demonstrated in dense cultures of algae during the exponential growth phase. Successful tolerance experiments were run using 50 ml cultures in 300 ml Bellco Hephallo flasks with 9 x 125 mm optically matched sidearms. Bristol's solution containing a known concentration of mercury and Bristol's with no mercury were added to the culture in different relative amounts to produce a final volume of 50 ml with equal culture density, generally about 0.15 optical density units in a 0.5 inch cell. Cultures having mercury concentrations of 0.5, 1.0, 2.0, and 3.0 ppm were maintained at 27 C under constant fluorescent light with an intensity of 400 ft. c. and swirled constantly on a Lab-line Shaker. To periodically estimate relative culture growth, the flasks were tilted, permitting the well-shaken suspension to flow into the sidearm. The sidearm was then inserted into a Bausch and Lomb Spectronic 20 spectrophotometer, and the optical density of the cultures was recorded at 700 mμ. Optical density is generally thought to be a reliable index of culture growth and relative cell number (Myers, 1962).

Where cultures showed no increase or decrease in optical density, subcultures were prepared with an inoculation of 1 drop of culture in 10 ml of mercury-free Bristol's to determine whether the inhibitory effect observed was of an algistatic or algicidal extent. An algistatic inhibition of the culture is indicated by retarded growth and reproduction capacity of cells for an indefinite

period of time. Growth would continue if the inhibitory substance were effectively removed. However, an algicidal condition indicates lethality. Microscopic analyses were also performed on cultures to detect immediate changes in reproductive rate and cellular condition.

Uptake studies

To determine the rate of mercury uptake by the test organisms and to discriminate between active and passive uptake, dense 275 ml cultures were examined. To a 250 ml culture of algae in a 500 ml Erlenmeyer flask, 25 ml of mercury-free Bristol's was added. Likewise, a killed control was prepared by adding 25 ml of 40 percent formaldehyde to another 250 ml of stock culture, thereby killing the cells yet maintaining the same density as the live culture.

To both cultures 1.0 ml of a 27.5 ppm mercury stock was added to bring the mercury concentration in each culture to 0.1 ppm. Immediately upon addition of the mercury, and periodically during incubation, three 10 ml aliquots of the live and the killed cultures were pipetted into separate 15 ml centrifuge tubes. From the centrifuge tubes, 1.0 ml of suspension was pipetted into 9 x 125 mm screw cap tubes. Suspensions were then centrifuged at full speed in an International clinical centrifuge and 1.0 ml of supernate from each centrifuge tube was likewise pipetted into screw cap tubes. Samples were sealed and frozen at -20 C until mercury analysis could be run on all samples. Control experiments showed that freezing and storage did not affect mercury concentration over a period of 3 weeks which was longer than any of the experimental cultures were stored.

These cultures were incubated in the same manner as the tolerance cultures. When cultures were sampled, the optical density was recorded in 0.5 inch cells at 700 mμ to estimate growth.

Analysis of mercury

A flameless atomic absorption spectrophotometer (Coleman Mas 50 mercury analyzer) was used for all analyses for mercury. The principle of the analytical method was thoroughly discussed by Hatch and Ott (1968), and the specific method outlined by the Coleman Instruments Division of Perkin Elmer Corporation (1970). The Coleman procedure for the analysis of mercury in various substances is to oxidize the samples with potassium permanganate to insure that all mercury present is in the mercuric ion state. After the sample is acidified with nitric and sulfuric acid and the excess permanganate removed with hydroxylamine hydrochloride, stannous chloride is added to reduce the mercury to its volatile metallic state. Air is bubbled into the sample and the mercury vapor passes into a flow-through absorption cell where it absorbs ultraviolet light at a wavelength of 253.7 namometers emitted by the mercury lamp. A phototube detects changes in transmittance. Standard 300 ml BOD bottles serve as reaction flasks for the Coleman Mas 50.

The method of preparation and analysis of mercury which was found to be workable for algal analysis represents a modified procedure outlined specifically for the analysis of urine samples (Coleman Instruments Division, 1971). The screw cap tubes were removed from the freezer and samples were allowed to reach room temperature. To each sample were added 5 ml of

35 percent nitric acid and 5 ml of 50 percent sulfuric acid. Samples were hydrolyzed in a water bath at 55 C for 20 minutes. No particulate matter could be observed in the hydrosylate when it was subsequently centrifuged and analyzed microscopically in control checks. Immediately after incubation in the water bath, the hydrosylate was transferred to 300 ml BOD bottles containing 10 ml of 6 percent potassium permanganate and approximately 70 ml of distilled water. About 5 ml of distilled water aided the complete transfer of the hydrosylate. To the oxidized samples, 20 ml of 1.5 percent hydroxylamine hydrochloride was added to decolorize the samples. After a period of one-half to one hour, the necessary reaction time predetermined in control experiments, 10 ml of 10 percent stannous chloride was added and the bubbler was immediately inserted into the BOD bottles. The highest deflection was recorded using the X-5 instrument mode.

Just prior to sample analysis, a standard curve was prepared. A standard 0.1 ppm mercury solution in mercury-free distilled water was prepared in the same manner as the cultures. From this standard, two standard replicates containing 0.00, 0.05, 0.10, 0.15, and 0.20 ppm mercury as mercuric chloride were prepared in culture tubes. Acids were added and standards were incubated and treated in every way as the samples were. Extra reagent blanks and 0.1 ppm standards were prepared and analyzed as controls during the time period of sample analyses. Statistical analysis of the standard curves revealed a coefficient of variation between 5 and 9 percent which was a major source of analytical variability in sample analysis.

Controls consisting of 1.0 ml of the original mercury-free stock culture were also hydrolyzed and analyzed to check for contamination and interfering substances. These analyses never showed detectable mercury concentrations. A control experiment was also run to establish whether a growing culture free of mercury produced any interfering metabolites over a period of time. All samples in these experiments assayed no detectable mercury.

RESULTS AND DISCUSSION

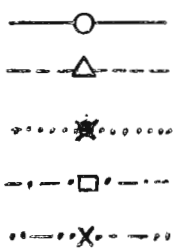
Tolerance of algae to mercury

Inhibition of small cultures and glass sorption. Because mercury was sorbed readily by test tube glass, many of the 10 ml cultures showed inconclusive data. Also, mercury concentrations were not high enough to show levels of mercury that were algicidal to the 1 drop inoculum. One drop of algal suspension taken from a culture with an optical density of 0.15 (approximated density of culture when used for inoculation) was 10^4 cells per ml. Figure 1 indicates the inhibitory effect of low concentrations of mercury on dual replicate cultures of Selenastrum capricornutum. The inhibitory effect was prolonged in media with higher levels of mercury; however, after 576 hours, growth was observed in all cultures. After initial inhibition, all cultures containing from 0.0 to 0.4 ppm mercury grew normally as reflected by their 96 to 144 hour lag phase followed by exponential growth. In contrast, the cultures containing 0.6 ppm mercury were markedly inhibited. Not only did they show no growth at all for 488 hours but maximum terminal growth reached only half that of the other mercury-containing cultures and controls.

Originally, the extended lag period of the cultures containing 0.6 ppm mercury was believed to be due to their inability to grow until a substantial amount of the mercury was removed through passive sorption by dead cells, allowing the remaining live cells to reproduce normally. This hypothesis was

Figure 1. Effect of mercury on growth in 1 drop inoculum of Selenastrum capricornutum.

Growth rate curves are shown for 10 ml cultures of Selenastrum capricorputum. Average optical density at 700 mμ in a 0.5 inch cell plotted against time after mercury addition reflects inhibition by various concentrations of mercury.



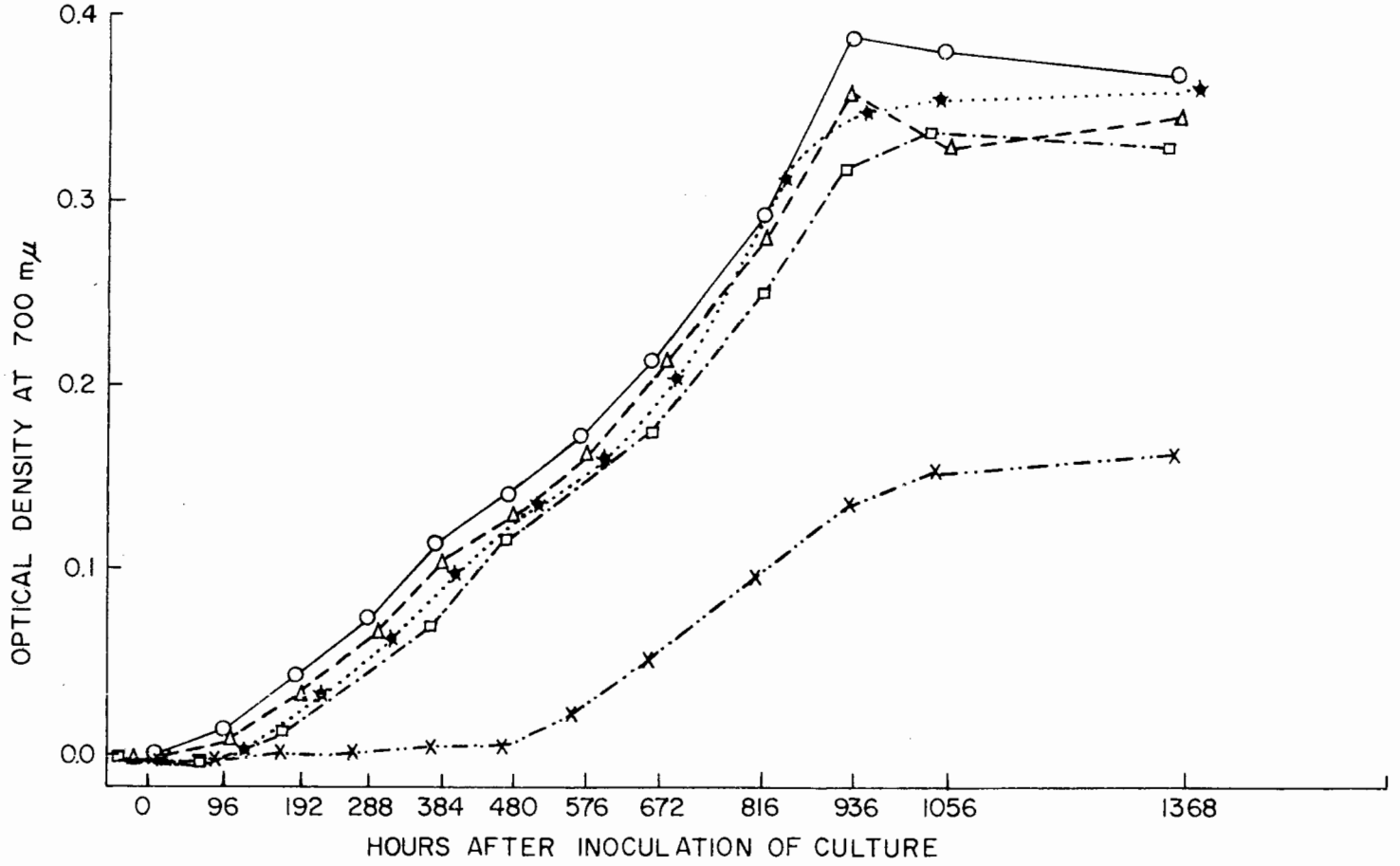
Bristol's medium with no mercury (control)

Bristol's medium with 0.1 ppm mercury

Bristol's medium with 0.2 ppm mercury

Bristol's medium with 0.4 ppm mercury

Bristol's medium with 0.6 ppm mercury


0.4
0.3
0.2
0.1
0.0
OPTICAL DENSITY AT 700 mμ
0
96
192
288
384
480
576
672
816
936
1056
1368
HOURS AFTER INOCULATION OF CULTURE

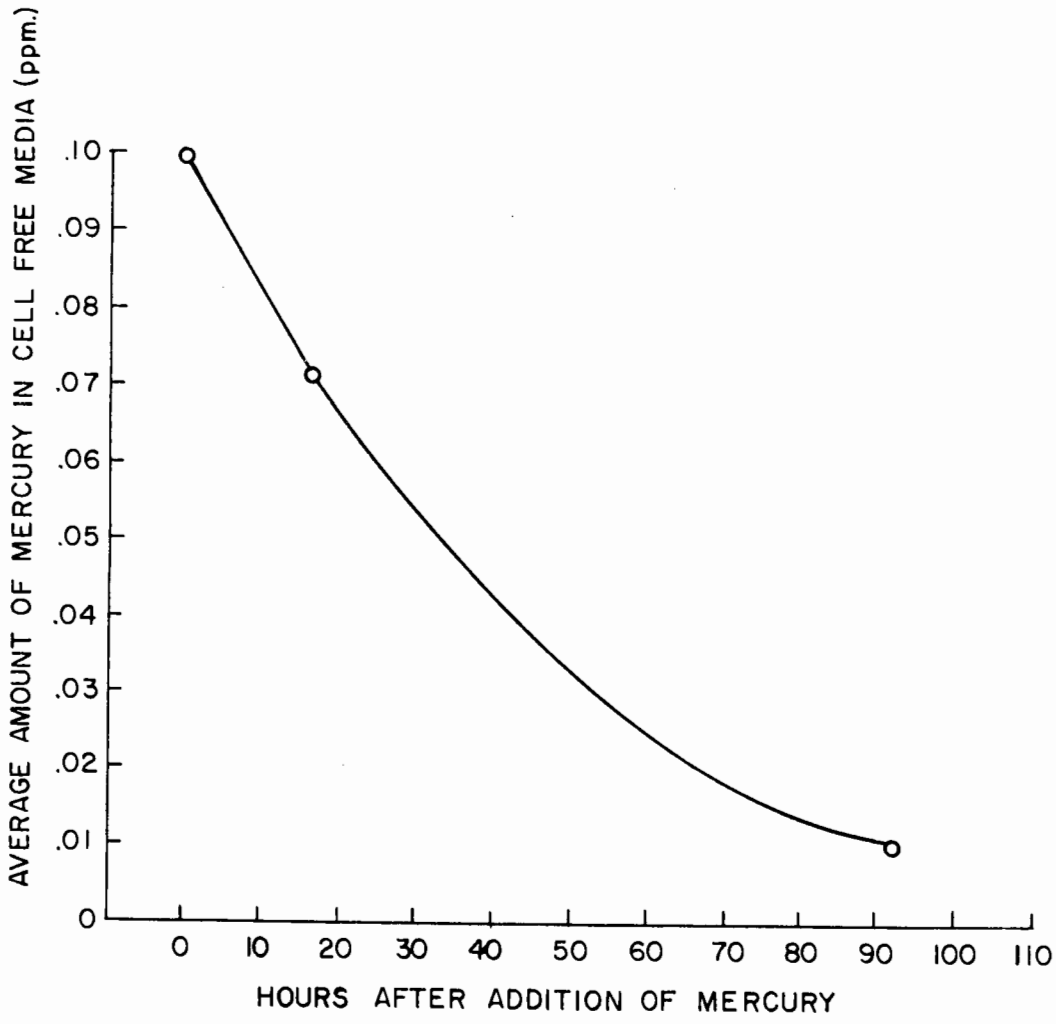
invalidated when the glass of the test tubes was shown to absorb mercury readily. An analysis of 25 day old cultures of Chlamydomonas reinhardi in media originally containing 0.1 ppm mercury showed that the media has lost approximately 90 percent of the initial mercury concentration. Analyses of the algae pellets accounted for only 0.5 percent of the initial mercury. However, when the pyrex test tubes were washed with concentrated nitric acid and the wash was analyzed, the needle deflected off the mercury analyzer scale, indicating that a great percentage of the mercury had been sorbed to the glass. Figure 2 shows the dynamics of the loss to culture flasks in cell-free sterile medium. Within 92 hours, 90 percent of the mercury originally in the medium was effectively removed by glass sorption, the same extent of loss shown by the 3 week old algae culture.

This problem of glass sorption was also encountered by Glooschenko (1969) who reported that up to 60 percent of the ^{203}Hg in his medium was sorbed within 22 hours by the walls of silicon glass containers. Glass sorption thus adequately explains the insignificant inhibition of algae growth by very low mercury concentrations. Since such a small algal inoculum was added, nothing prevented quick sorption which in turn permitted uninhibited growth.

However, the cultures containing 0.6 ppm mercury probably provided enough unabsorbed mercury in solution after glass sorption to impair the growth of the algae cells. Additionally, the relatively low final algal density probably reflected some exchange of merucry between the test tube walls and the algae, manifesting a prolonged inhibition. Very similar results were obtained in long term 1 drop inoculation cultures of Chlamydomonas reinhardi

Figure 2. Mercury sorption by pyrex culture tubes.

Mercury content of sterile Bristol's medium with an initial concentration of 0.1 ppm mercury plotted against time after addition of mercury. Points reflect an average of three replicate analyses at each sampling. Note the steady loss due to glass sorption.

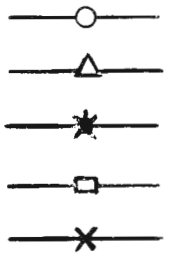

AVERAGE AMOUNT OF MERCURY IN CELL FREE MEDIA (ppm.)
.10
.09
.08
.07
.06
.05
.04
.03
.02
.01
0
0
10
20
30
40
50
60
70
80
90
100
110
HOURS AFTER ADDITION OF MERCURY

and Chlorella pyrenoidosa. These results indicated a need for shorter term experiments with an initially heavy inoculum of culture to minimize the glass sorption problem.

Lethal and inhibitory levels of mercury in large cultures. Using 300 ml flasks with sidearms the inhibitory effect of mercury to initially heavy, exponentially growing cultures of Chlorella pyrenoidosa and Selenastrum capricornutum could be successfully studied. Figure 3 shows the effect of various concentrations of mercury on C. pyrenoidosa. Optical densities taken from the sidearm reflected relative culture growth. The control shows steady growth, yet the growth rate may have been slightly restrained since only about 100 ml of fresh media was added to 400 ml of dense culture. Because this 500 ml stock served as a source of inoculum for all dilutions with mercury, all growth rate curves in Figure 3 are comparable. The 0.5 ppm culture showed the expected slight inhibition of growth, but after 72 hours grew at approximately the same rate as the control and surpassed the control in culture density after 240 hours. A decrease in optical density in the 1.0, 2.0, and 3.0 ppm cultures was noted, and these cultures were visibly bleached after 24 hours. Microscopic analyses revealed that most cells were in some stage of division but were quite pale. This depression of chlorophyll content was assumed to be due to the suppression of chlorophyll producing enzymes by mercury as described by Matson et al. (1972). Optical density continued to decline in all three cultures until 240 hours had elapsed. After this time, the 1.0 and 2.0 ppm cultures began to visibly regain their normal green pigmentation and shortly thereafter showed a steadily

Figure 3. Effect of mercury on growth of dense cultures of Chlorella pyrenoidosa.

Inhibition of dense 50 ml cultures of Chlorella pyrenoidosa by various concentrations of mercury is shown. Cultures were in 300 ml flasks equipped with optically matched sidearms. Optical density at 700 mμ in a 0.5 inch cell plotted against time after mercury addition reflects relative growth for individual cultures at each mercury concentration. Lethal concentration of mercury was 3 ppm.



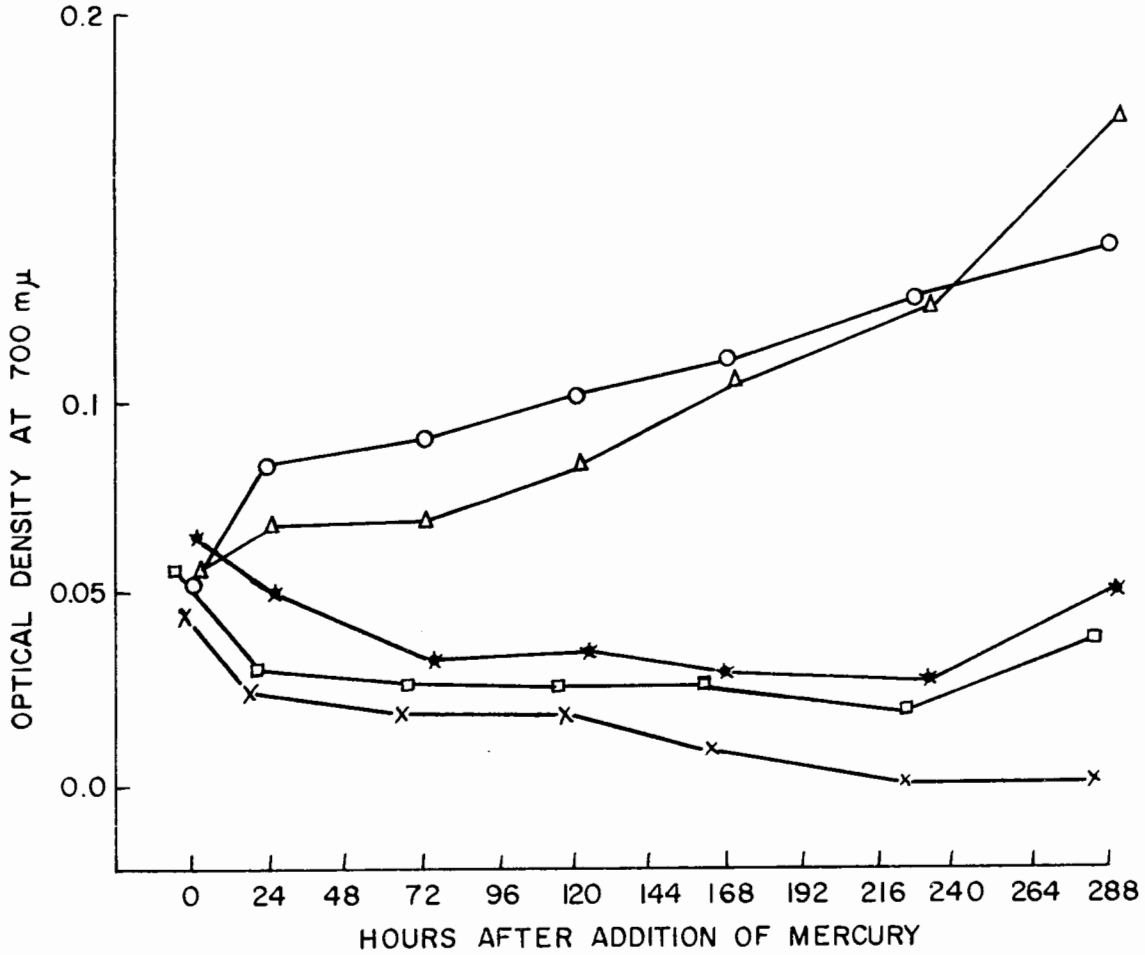
Bristol's medium with no mercury (control)

Bristol's medium with 0.5 ppm mercury

Bristol's medium with 1.0 ppm mercury

Bristol's medium with 2.0 ppm mercury

Bristol's medium with 3.0 ppm mercury


0.2
0.1
0.05
0.0
OPTICAL DENSITY AT 700 mμ
0
24
48
72
96
120
144
168
192
216
240
264
288
HOURS AFTER ADDITION OF MERCURY

increasing density. Though only one data point reflected this increased culture growth, cultures were maintained for a week and further growth was visibly evident. Evidently glass sorption of mercury permitted growth in previously algistatic 1.0 and 2.0 ppm cultures. The culture containing 3.0 ppm mercury showed no growth; apparently 3.0 ppm mercury was lethal to this culture. Microscopic analysis of the culture after 14 days revealed that cells, although bleached, had maintained their structural integrity. The 10 ml subcultures made after 24 hours incubation in mercury-free media showed growth from the 1.0 and 2.0 ppm cultures, further confirming the algistatic effect of the mercury concentrations employed. Subcultures from the 3.0 ppm culture showed no growth, confirming the lethality of mercury at this concentration.

Selenastrum capricornutum was shown to exhibit similar inhibition. Figure 4 shows that 0.5 ppm mercury slightly inhibits growth relative to the control. As reflected by declines in optical densities the 1.0, 2.0, and 3.0 ppm cultures were more markedly inhibited. After 240 hours, the algistatic effects of the mercury had ceased, probably due to glass sorption, and cultures grew steadily. As expected all subcultures in mercury-free media grew. No lethal concentration of mercury could be established, but replicate cultures showed that 7.0 ppm was lethal to similar algae concentrations of S. capricornutum.

To test the hypothesis that well nourished algae would be more tolerant to mercury, a comparison was made between cultures in protein-rich media and minimal-nutrient (Bristol's) media. Both kinds of cultures contained the same quantity of algae and the same concentration of mercury. A series of mercury

Figure 4. Effect of mercury on dense cultures of Selenastrum capricornutum.

Growth rate curves are shown for individual dense 50 ml cultures of Selenastrum capricornutum. Optical density at 700 mμ in a 0.5 inch cell plotted against time after mercury addition reflects inhibition by various concentrations of mercury.

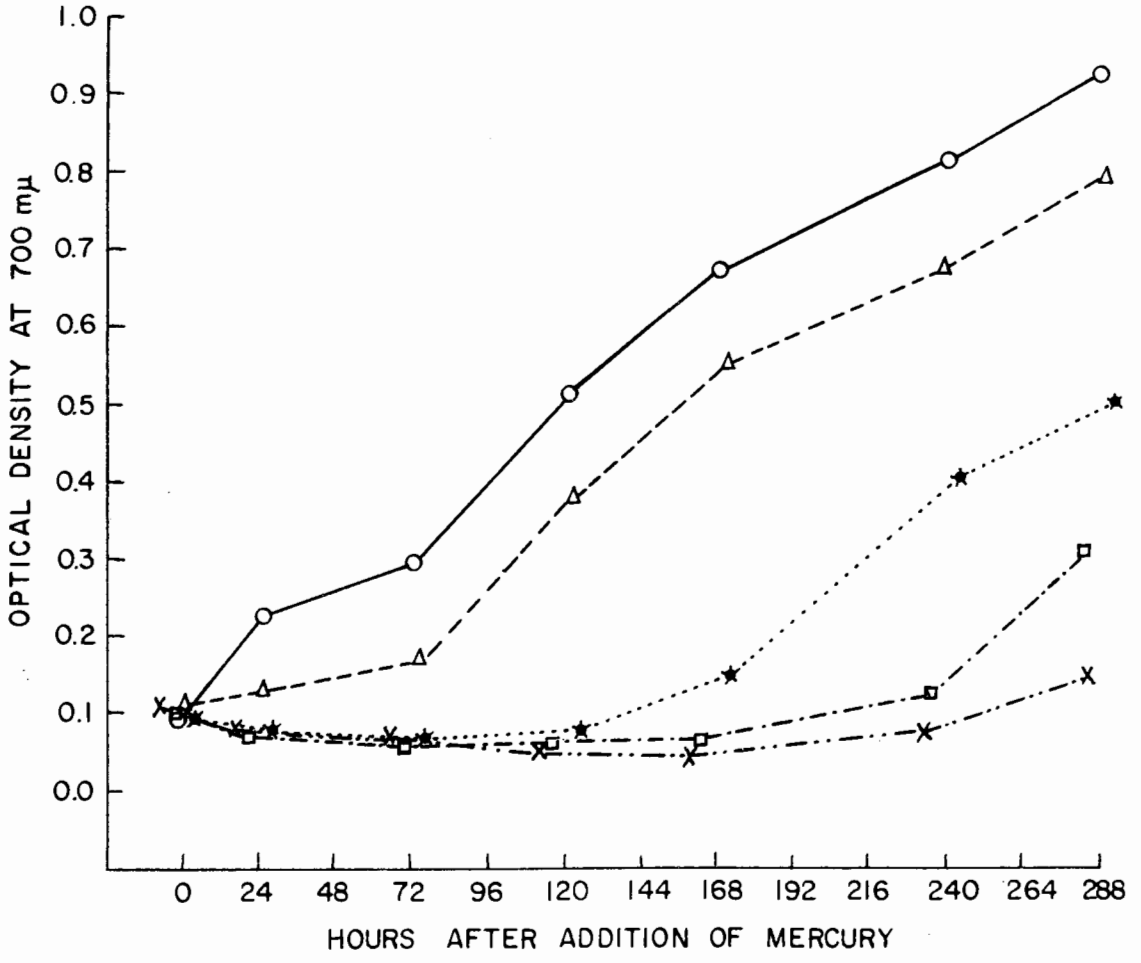
Bristol's medium with no mercury (control)

Bristol's medium with 0.5 ppm mercury

Bristol's medium with 1.0 ppm mercury

Bristol's medium with 2.0 ppm mercury

Bristol's medium with 3.0 ppm mercury


OPTICAL DENSITY AT 700 mμ
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
24
48
72
96
120
144
168
192
216
240
264
288
HOURS AFTER ADDITION OF MERCURY

concentrations ranging from 0-50 ppm were prepared in both Bristol's and proteose media (Starr, 1964; see Appendix). In Bristol's medium, Chlorella pyrenoidosa was tolerant to 2.0 ppm mercury while 3.0 ppm proved lethal, whereas in nutrient-rich proteose medium it was tolerant to 10 ppm mercury but 50 ppm proved lethal. Therefore, this species of algae may have a greater tolerance to mercury in nutrient-rich media, or these results may be caused by the binding of the mercury to sulfhydryl groups of the proteins in the nutrient-rich medium.

Because substantial mercury sorption to pyrex glass was noted in this investigation and silicon glass sorption was noted by Glooschenko (1969), and because different media with the same mercury concentration demonstrated different levels of toxicity, the nature of the natural or artificial environment may profoundly affect the level of mercury toxicity. If the mechanism of mercury sorption by algae is predominantly passive, the ratio between the volume of the media and the surface area of the algal cell walls and the container might prove to be critical to the inhibitory response. Likewise, the chemical makeup of the media, the algae, and the confines may also be important to the toxicity levels. Since silicon seems to readily sorb mercury, the diatoms with their silicon cell walls might be expected to sorb mercury more readily than other algae. Materials in the bottom sediments such as quartz and biotic constituents such as bacteria may also afford sorption sites. Preferential chemical binding to the sulfur-containing sediments is also an important factor in assessing the availability of mercury to overlying water (Feick et al., 1972).

Chemical constituents in the medium are probably equally important contributors to the level of toxicity. The increased detectable mercury in relatively saline environments discussed by Feick et al. (1972) indicates that algae might be more susceptible to mercury where salt concentrations are high. Ethylenediamine tetracetic acid (EDTA) used as a chelating agent in the preparation of Bristol's medium (see Appendix) may have established an equilibrium with the sorption capacity of the algae. Because chemical binding may occur between mercury and some chelating constituent of the medium, a decreased availability of mercury to the algae may result.

Uptake of mercury

Mathematical determination of percent uptake. To determine whether mercury was being actively sorbed by algae, cultures were prepared containing 0.1 ppm mercury as mercuric chloride. Subsequent mercury analyses of 1.0 ml subsamples of algal suspensions and 1.0 ml subsamples of the supernatant media after centrifugation of three 10 ml samples demonstrated the amount of mercury sorbed by the cells by taking the difference between these values.

To show whether uptake was an active or passive process, live cultures and formalin-killed cultures were incubated and analyzed simultaneously. Mercury uptake by both was then compared. Although each culture was prepared in the same way, the formalin-killed culture generally registered more mercury. Also the sorption of mercury in the media, particularly in the killed culture, indicated a slight decline in the mercury concentration of the medium with time due to glass sorption. For these reasons, the absolute

quantity of mercury sorbed by the cells was an unreliable index of relative mercury uptake. Percent uptake was therefore determined by comparing the relative amount of mercury in the cells to the amount of mercury in the suspension at any given time. By accounting for concentration differences and glass sorption, many statistical inaccuracies were eliminated. Percent uptake was a more accurate comparison between mercury sorption by the live and the killed cultures.

Mercury uptake by Selenastrum capricornutum. Uptake studies were first made on Selenastrum capricornutum using three cultures of the same density--one live, one killed, and one live culture with the vessel covered with opaque tape to prevent light penetration. Figure 5 shows the percent uptake of mercury by each of the three cultures over the 2 hour experiment period. Analysis of the three replicate samples of each culture demonstrated that an average of 39 percent of the mercury available was sorbed by cells immediately. The amount sorbed increased slightly after 1 hour to 39 percent and to 41 percent after 2 hours. Uptake observed in the killed cells was only slightly less than live uptake. Mercury uptake by dark-incubated cultures was somewhat less initially, but the relatively high standard deviation reflected some analytical error. Within 1 hour the percent uptake by the dark-incubated culture resembled the uptake of the other two cultures and the final uptake was nearly identical.

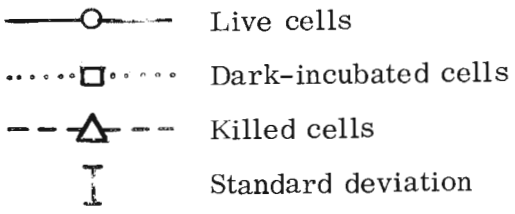
The inconsistent, sometimes high standard devitation demonstrated, especially by the 2 hour analysis, was observed throughout most uptake analyses. Much of the analytical error seemed unavoidable. The coefficient

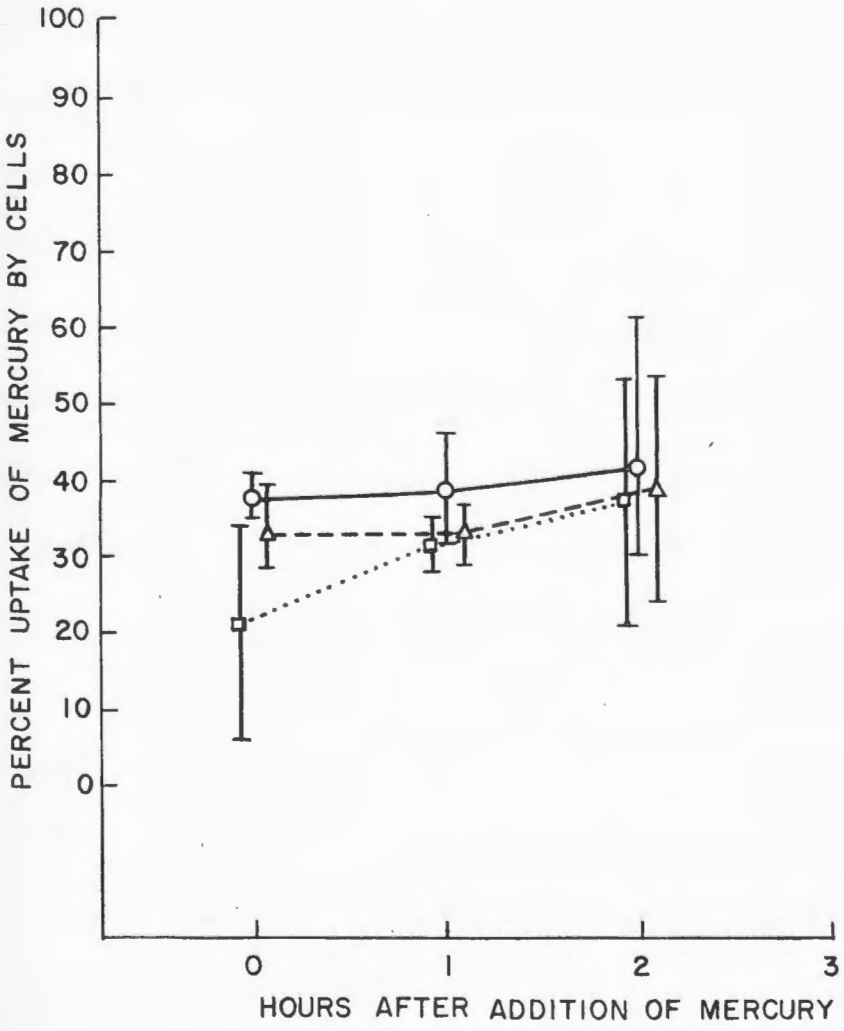
Figure 5. Mercury uptake by Selenastrum capricornutum.

Percent uptake of mercury by Selenastrum capricornutum cells over a 2 hour period plotted against time after mercury addition. Points are based on an average of three replicate analyses for mercury in the suspension and the supernate from a live culture, a dark-incubated culture, and a formalin-killed culture all of the same initial density.

$$\text{Percent uptake of mercury by cells} = \frac{\text{Average mercury concentration in cells at time t}}{\text{Average mercury concentration in suspension at time t}} \times 100$$

Percent mercury uptake:




PERCENT UPTAKE OF MERCURY BY CELLS
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
HOURS AFTER ADDITION OF MERCURY

of variation of 5-9 percent inherent in the standard curve could account for standard deviations of up to 20 percent mercury uptake by cells. The total error is assumed to be the sum of the instrumental error shown by the variability within the standard curve, biological and chemical variability among cultures, and any additional errors which seem inherent when quantitative analyses are made in the region of 0.1 ppm. In the analysis of data, these sources of error should be considered.

In this statistical analysis, t-tests employed compare the mean percent mercury uptake of two samples. The test considers the variance of the mean and thus discloses any overlap in this statistic reflected by a nonsignificant difference between the means. If a significant difference is shown at the 0.05 confidence level, the investigator is wrong 1 in 20 times when he hypothesizes that the means are different. If a significant difference is shown at the 0.01 level, chances that he is wrong in the hypothesis are 1 in 100. The probability (P) of error is less than or equal to 0.05 and 0.01 respectively.

Statistical analyses of the percent mercury uptake by these cultures of S. capricornutum shown in Table 1 reveal that the cultures are statistically identical. T-tests comparing the mean percent uptake of the live and the killed cells at each time demonstrate no significant difference at the 0.05 level. By comparing the mean percent uptake by the light-live culture to that of the dark-incubated culture, a significant difference is shown at the 0.05 level at the 1 hour interval; no significant difference is shown at the 0.01 level.

Table 1. Comparison of mercury uptake in cultures of Selenastrum capricornutum. T-tests comparing the mean percent uptake of mercury by live cultures of Selenastrum capricornutum to the mean percent uptake of the killed and the dark-incubated cultures at sampling intervals after mercury addition over the 2 hour period.

Time in hours after mercury addition	Comparison	t value
0	$\bar{X}_{live}$ vs. $\bar{X}_{killed}$	0.871*
1	$\bar{X}_{live}$ vs. $\bar{X}_{killed}$	2.034*
2	$\bar{X}_{live}$ vs. $\bar{X}_{killed}$	0.211*
0	$\bar{X}_{live}$ vs. $\bar{X}_{dark}$	1.986*
1	$\bar{X}_{live}$ vs. $\bar{X}_{dark}$	2.809**
2	$\bar{X}_{live}$ vs. $\bar{X}_{dark}$	0.334*

Degrees of freedom: 4
Tabular t value at the 0.05 level: 2.776

*Not significant at the 0.05 level, $P \leq 0.05$.
**Significant at the 0.05 level.

Because of the statistically equal extent of uptake in the live and the killed cultures, no measurable active uptake by S. capricornutum occurred in the 2 hour time span. The mechanism of uptake seemed to be solely of a passive-sorptive nature.

The dark-incubated culture demonstrated nearly the same uptake as the light-live culture; mercury uptake thus seemed independent of light. Glooschenko (1969) also found an insignificant difference in ^{203}Hg uptake between light-live and dark-incubated cultures of Chaetoceros costatum, a marine diatom. Evidence indicated that dark controls were therefore not required in further uptake analyses.

Table 2 shows t-tests which compare the mean uptake of each culture at 1 and 2 hours with the mean uptake at time 0. Over a 2 hour period the percent uptake does not change significantly from immediate uptake.

Table 2. Comparison of subsequent mercury uptake in Selenastrum capricornutum. T-tests comparing the mean percent uptake of Selenastrum capricornutum at the 1 and 2 hour sampling intervals with the mean uptake at time 0.

Culture type	Comparison	t value
Live	$\bar{X}_{1\text{ hr.}}$ vs. $\bar{X}_{0\text{ hr.}}$	1.012*
Live	$\bar{X}_{2\text{ hr.}}$ vs. $\bar{X}_{0\text{ hr.}}$	0.520*
Dark	$\bar{X}_{1\text{ hr.}}$ vs. $\bar{X}_{0\text{ hr.}}$	1.326
Dark	$\bar{X}_{2\text{ hr.}}$ vs. $\bar{X}_{0\text{ hr.}}$	1.337*
Killed	$\bar{X}_{1\text{ hr.}}$ vs. $\bar{X}_{0\text{ hr.}}$	0.158*
Killed	$\bar{X}_{2\text{ hr.}}$ vs. $\bar{X}_{0\text{ hr.}}$	0.526*

Degrees of freedom: 4
Tabular t value at the 0.05 level: 2.776

*Not significant at the 0.05 level, $P \leq 0.05$.

An attempt was made in another experiment with S. capricornutum to describe detectable differences in the magnitude of mercury uptake with varying concentrations of algae. A stock culture was diluted by one-third and two-thirds with fresh media, reducing the originally dense culture which registered an optical density of 0.260 in a 0.5 inch cell to 0.190 and 0.090 in the diluted cultures. Mercuric chloride stock was then added making the concentration of mercury in each culture 0.1 ppm.

After 1 hour, the three cultures were sampled as before and aliquots of the suspension and supernate were analyzed for mercury. Percent uptake of mercury for each dilution is shown in Figure 6. The 34 percent mercury uptake in the most dense culture agrees closely with the fraction of the mercury sorbed in the previous uptake study on S. capricornutum. Increased culture density and hence greater numbers of algal cells per culture should be reflected by an increase in percent mercury uptake since more cells would increase surface area available for mercury sorption. No statistically significant difference is shown at the 0.05 level among the three mean percent mercury uptakes due to the high cell number per culture and the lack of precision in analyzing minute concentrations of mercury.

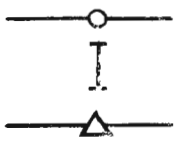
A control experiment revealed that by using higher mercury concentrations and relying on the X-1 mode of the mercury analyzer, the analytical precision was not improved. An experiment using more cultures with a greater spread in culture densities might have shown significant difference in uptake, but in very dilute cultures the variance between the number of cells in two cultures of the same dilution might be very great, compounding the analytical error.

Mercury uptake by Chlamydomonas reinhardi. Cultures of Chlamydomonas reinhardi were investigated for mercury uptake. Cellular and mercury concentrations were equal in the live and the killed cultures, and preparation, incubation, sampling, and analysis were done as previously described; however, this particular experiment consisted of a 49 hour period

Figure 6. Effect of culture density on mercury uptake in Selenastrum capricornutum.

Percent uptake of mercury by live Selenastrum capricornutum cells 1 hour after mercury addition is plotted against the relative concentration of algae from a stock culture and against the optical density at 700 mμ in a 0.5 inch cell. Points are based on an average of three replicate analyses for mercury in the suspension and the supernate from each culture.

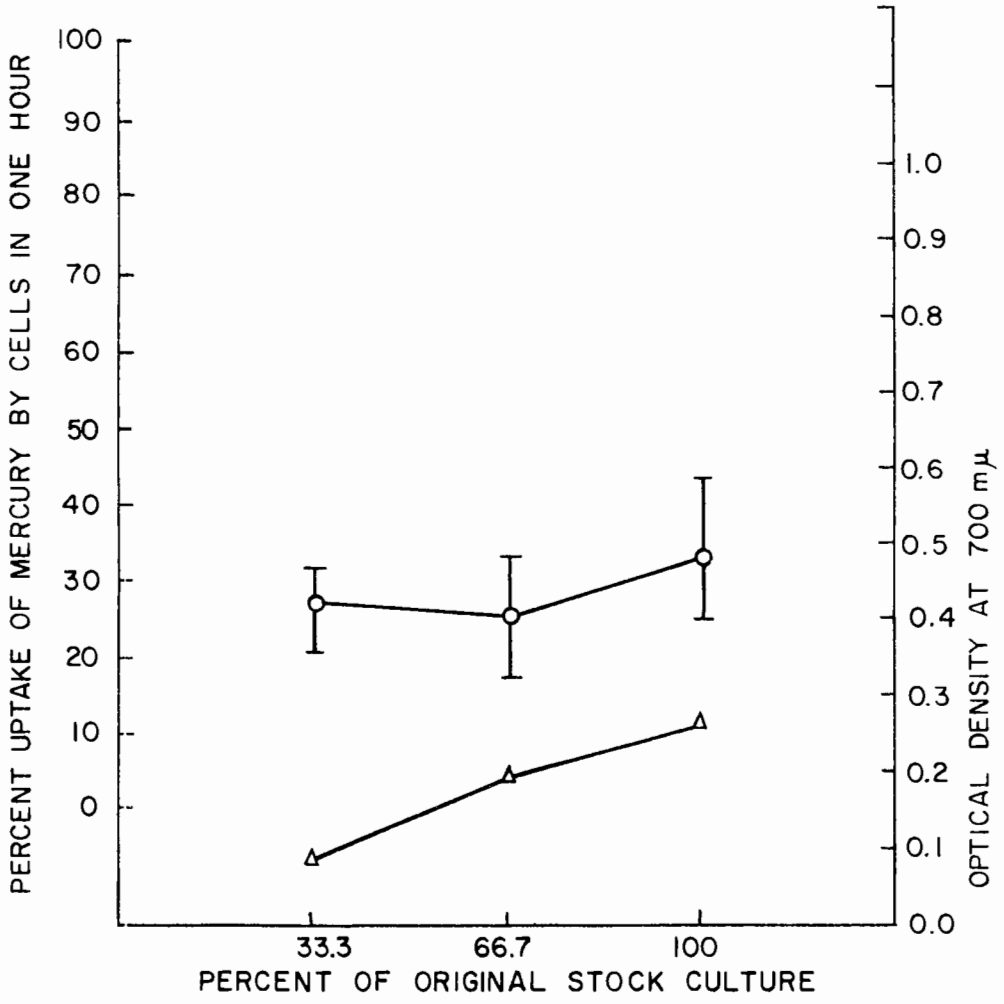
$$\text{Percent uptake of mercury by cells in 1 hour} = \frac{\text{Average mercury concentration in cells after 1 hour}}{\text{Average mercury concentration in suspension after 1 hour}} \times 100$$



Percent mercury uptake by live cells

Standard deviation

Optical density at 700 mμ in a 0.5 inch cell


PERCENT UPTAKE OF MERCURY BY CELLS IN ONE HOUR
100
90
80
70
60
50
40
30
20
10
0
OPTICAL DENSITY AT 700 mμ
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
33.3
66.7
100
PERCENT OF ORIGINAL STOCK CULTURE

with five sampling intervals. The optical density of both live and formalin killed cultures was initially 0.15. Erratic instrument deflection in the analysis of the first samples of algae resulted in lost data. Percent uptake by live cultures shown in Figure 7 reflected consistent and reasonable concentrations of mercury after the erroneous 0 and 1 hour readings. Also, additional standards showed proper deflections.

As demonstrated by Figure 7, mercury uptake by live cells and relatively low yet constant throughout the incubation period. Statistical analyses shown in Table 3 demonstrated no significant difference in the available percentages. The killed cells showed a substantial uptake of 18 percent immediately, 24 percent after 1 hour, and 34 percent after 7 hours. As Table 4 shows, the killed uptake is significantly higher than the live uptake at 7 hours. The reading of 58 percent uptake by killed cells at 22 hours is also significantly higher than live percent uptake at that time. A significant difference is not shown between final killed and final live uptake. Because the percent mercury uptake by killed cells is higher at all points than uptake by live cells, active uptake did not occur. In fact, because mercury uptake was significantly higher in killed cells during two intervals, living C. reinhardi cells in some way resist mercury uptake for a time. Such resistance is not shown in other uptake studies and the stock culture of C. reinhardi was lost before the experiment could be repeated.

Short term mercury uptake by Chlorella pyrenoidosa. Uptake of mercury by Chlorella pyrenoidosa was studied in depth with short and long term experiments. The short term experiment was conducted in a 107 hour period

Figure 7. Mercury uptake by Chlamydomonas reinhardi.

Percent uptake by Chlamydomonas reinhardi cells over a 49 hour period is plotted against time after mercury addition. Points are based on an average of three replicate analyses for mercury in the suspension and the supernate from the live and the formalin-killed culture of the same initial density.

$$\text{Percent uptake of mercury by cells} = \frac{\text{Average mercury concentration in cells at time t}}{\text{Average mercury concentration in suspension at time t}} \times 100$$

Percent mercury uptake:

—○— Live cells

—△— Killed cells

I Standard deviation

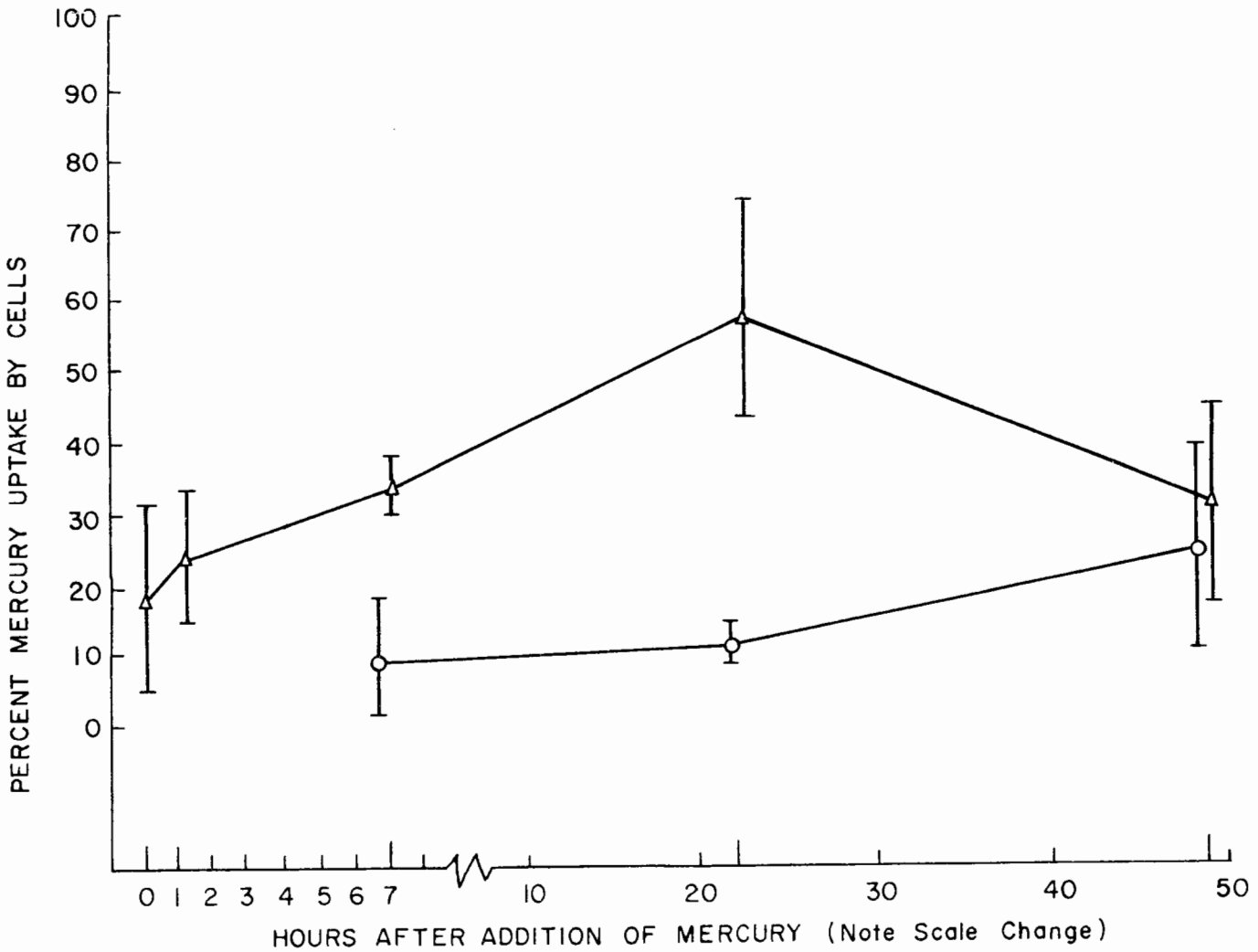

PERCENT MERCURY UPTAKE BY CELLS
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
10
20
30
40
50
HOURS AFTER ADDITION OF MERCURY (Note Scale Change)

Table 3. Comparison of mercury uptake in Chlamydomonas reinhardi. T-tests comparing the mean percent mercury uptake for both the live and the killed cultures of Chlamydomonas reinhardi at the 22 and 49 hour intervals with the mean uptake at 7 hours.

Culture type	Comparison	T value
Live	$\bar{X}_{22}$ hrs. vs. $\bar{X}_{7}$ hrs.	0.489*
Live	$\bar{X}_{49}$ hrs. vs. $\bar{X}_{7}$ hrs.	1.485*
Killed	$\bar{X}_{22}$ hrs. vs. $\bar{X}_{7}$ hrs.	2.525*
Killed	$\bar{X}_{29}$ hrs. vs. $\bar{X}_{7}$ hrs.	0.448*

Degrees of freedom: 4
Tabular t value at the 0.05 level: 2.776

*Not significant at the 0.05 level, $P \leq 0.05$.

Table 4. Comparison of subsequent mercury uptake in Chlamydomonas reinhardi. T-tests comparing the mean percent mercury uptake by the live cultures of Chlamydomonas reinhardi to that of the killed cultures at sampling intervals of 7, 22, and 49 hours.

Time in hours after mercury addition	Comparison	t value
7	$\bar{X}_{\text{live}}$ vs. $\bar{X}_{\text{killed}}$	4.424*
22	$\bar{X}_{\text{live}}$ vs. $\bar{X}_{\text{killed}}$	4.829**
49	$\bar{X}_{\text{live}}$ vs. $\bar{X}_{\text{killed}}$	0.567***

Degrees of freedom: 4
Tabular t value at 0.05 level: 2.776
Tabular t value of 0.01 level: 4.604

*Significant at 0.05 level, $P \leq 0.05$.
**Significant at the 0.01 level, $P < 0.01$.
***Not significant at the 0.05 level.

with five sampling intervals. The live culture and the killed control culture were prepared, incubated, sampled, and analyzed for mercury as before. In addition, at each sampling interval the live and the killed cultures were analyzed for growth using optical density.

Cellular percent uptake of mercury and optical density plotted against time after mercury addition is shown in Figure 8. Mercury uptake by the live cells was immediately substantial at 23 percent. Judging from the overlap of standard deviations and the statistically insignificant difference between the 0 and 1 hour analyses, the low mean uptake at 1 hour was probably due to analytical error. Also, because analytical difficulties negated the time 0 killed uptake reading, Tables 5 and 6 reflect data from the 26, 86, and 107 hour samples only. A slight increase was noted in the culture density at 26 hours, but the mean uptake was nearly the same as that registered at the time 0 sample. A slight increase was noted in percent uptake from 27 to 30 percent while optical density increased from 0.06 to 0.10 during the period from 26 to 86 hours. As culture density increased to an optical density of 0.18 at 107 hours, no significant difference in percent uptake was shown.

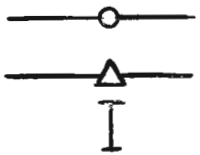
The percent mercury uptake by killed cells noted in Figure 8 is greater than or equal to the live uptake in all samples except at 107 hours. Mercury uptake by killed cells was found to be insignificantly different from that by live cells at all intervals including the 107 hour sampling. No active sorption of mercury was observed.

Figure 8. Mercury uptake by Chlorella pyrenoidosa (short term experiment).

Percent uptake of mercury by Chlorella pyrenoidosa cells over a 107 hour period is plotted against time after mercury addition. Points are based on an average of three replicate analyses for mercury in the suspension and the supernate sample from a live culture and a formalin-killed culture of the same initial density. Growth is reflected by optical density at 700 mμ in a 0.5 inch cell as shown.

$$\text{Percent uptake of mercury by cells} = \frac{\text{Average mercury concentration in cells at time t}}{\text{Average mercury concentration in suspension at time t}} \times 100$$

Percent mercury uptake:



Optical density at 700 mμ in a 0.5 inch cell:

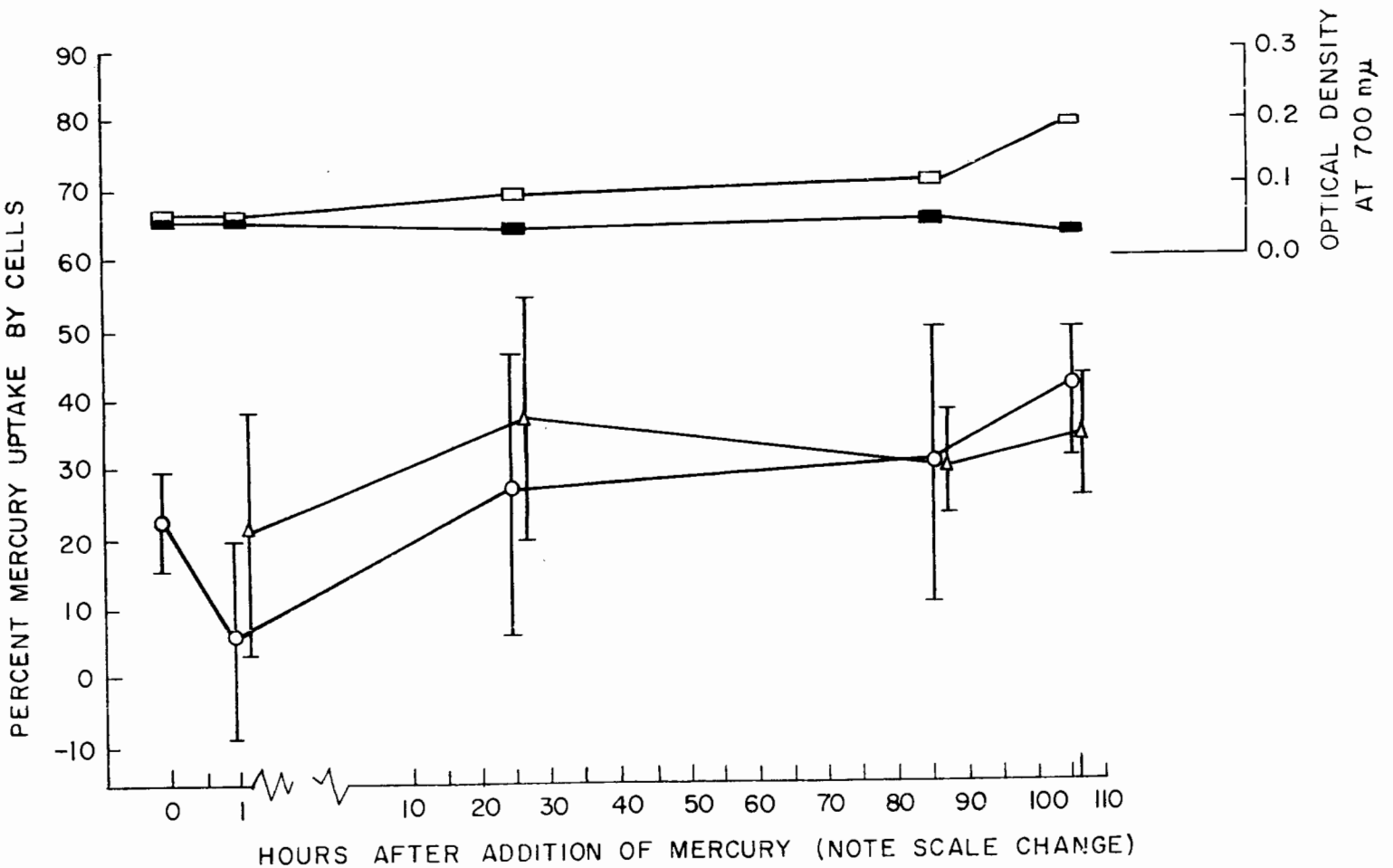

PERCENT MERCURY UPTAKE BY CELLS
90
80
70
60
50
40
30
20
10
0
-10
0
1
10
20
30
40
50
60
70
80
90
100
110
HOURS AFTER ADDITION OF MERCURY (NOTE SCALE CHANGE)
0.3
0.2
0.1
0.0
OPTICAL DENSITY
AT 700 mμ

Table 5. Comparison of subsequent mercury uptake by Chlorella pyrenoidosa (short term experiment). T-tests comparing the mean percent mercury uptake by Chlorella pyrenoidosa as the 86 and 107 hour intervals with the mean percent uptake at 26 hours for both the live and the killed cultures.

Culture type	Comparison	t value
Live	$\bar{X}_{86}$ hrs. vs. $\bar{X}_{26}$ hrs.	0.2542*
Live	$\bar{X}_{107}$ hrs. vs. $\bar{X}_{26}$ hrs.	1.1662*
Killed	$\bar{X}_{86}$ hrs. vs. $\bar{X}_{26}$ hrs.	0.5732*
Killed	$\bar{X}_{107}$ hrs. vs. $\bar{X}_{26}$ hrs.	0.2495*

Degrees of freedom: 4
Tabular t value at the 0.05 level: 2.776

*Not significant at the 0.05 level, $P < 0.05$.

Table 6. Rate of mercury uptake by Chlorella pyrenoidosa (short term experiment). Rate of mercury uptake by Chlorella pyrenoidosa expressed as the mean percent uptake of three replicates per optical density unit for live cultures over the 107 hour period.

Time in hours after mercury addition	Mean percent uptake	Optical density	Rate of uptake percent/O. D. units
0	23	0.055	413
26	27	0.060	450
86	31	0.100	310
107	41	0.180	228

Table 5 demonstrates that in both the live and the killed cultures no significant difference was shown between the mercury uptake in successive samples. Additionally, since no statistical difference was shown between the final live uptake and the 1 hour killed uptake (initial uptake having been lost), neither the live nor the killed cultures showed any significant changes in uptake over the 107 hour period.

The rate of mercury uptake expressed as mean percent uptake per optical density unit is shown in Table 6. The rate increase between the initial uptake and the uptake recorded at 26 hours is due to a slight rise in percent mercury uptake with a relatively small change in optical density. The steady decline in the rate of mercury uptake after 26 hours indicated biological dilution by rapidly increasing cell number and culture density. Had the rate of uptake increased as optical density increased, active transport of mercury might have been indicated. However, such was not the case.

Over the 107 hour incubation period, less than 10 percent of the mercury initially added was lost to the glass in both the live and the killed cultures. Apparently the algal cells compete favorably with the glass for mercury in initially dense cultures. The glass sorption problem was minimized principally by the use of initially dense cultures, and a longer term uptake study was run using C. pyrenoidosa.

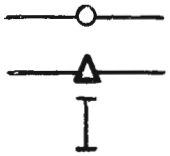
Long term mercury uptake by Chlorella pyrenoidosa. Cultures were treated as before, and seven samples of three replicates each were analyzed for mercury and optical density over a 12 day period. As shown in Figure 9,

Figure 9. Mercury uptake by Chlorella pyrenoidosa (long term experiment).

Percent uptake of mercury by Chlorella pyrenoidosa cells over a 288 hour (12 day) period is plotted against time after mercury addition. Points are based on an average of three replicate analyses for mercury in the suspension and the supernate from a live culture and a formalin-killed culture of the same initial density. Culture growth is reflected by optical density at 700 mμ in a 0.5 inch cell as shown.

$$\text{Percent uptake of mercury by cells} = \frac{\text{Average mercury concentration in cells at time t}}{\text{Average mercury concentration in suspension at time t}} \times 100$$

Percent mercury uptake:

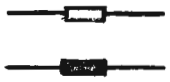


Live cells

Killed cells

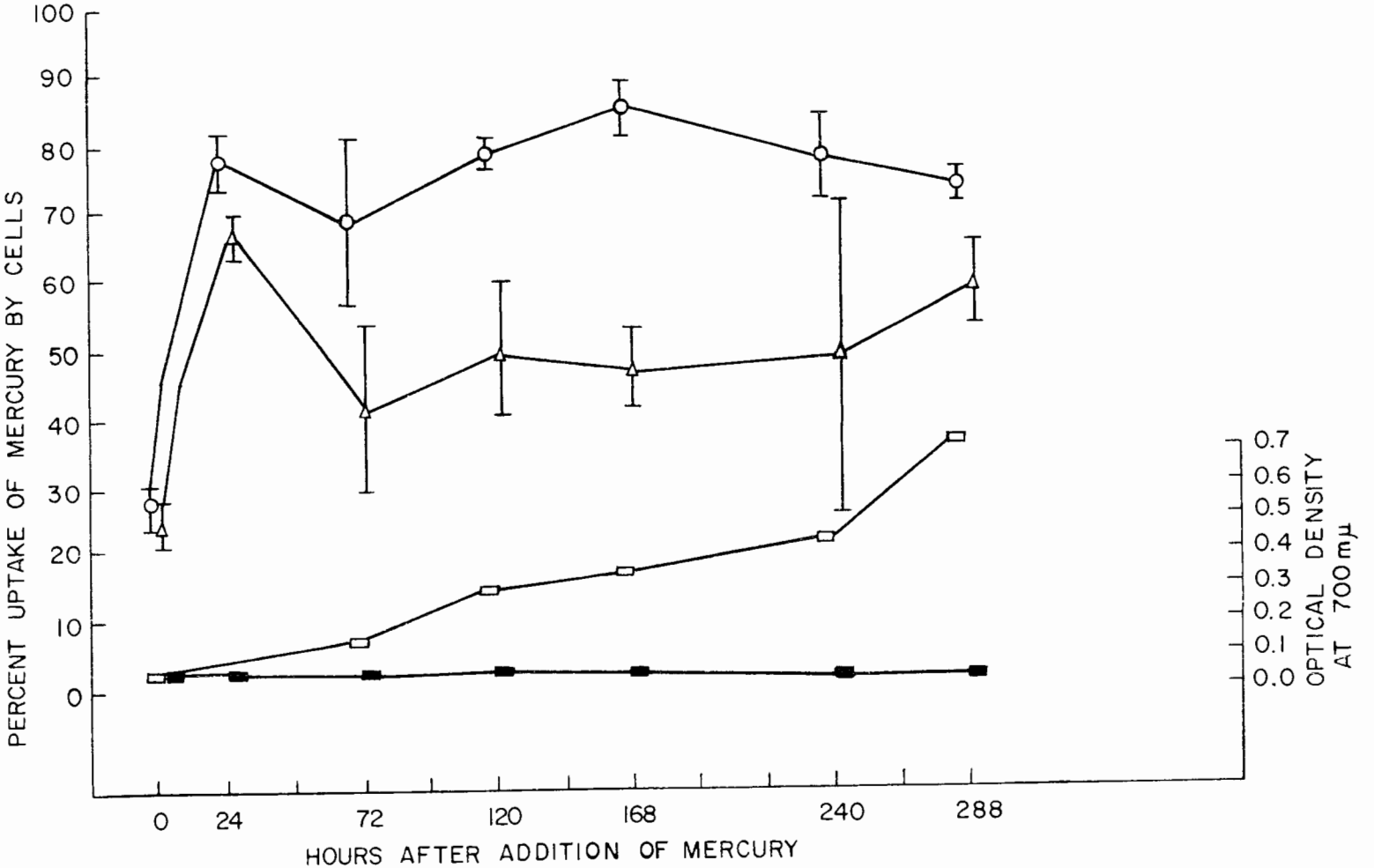
Standard deviation

Optical density at 700 mμ in a 0.5 inch cell:



Live culture

Killed culture


PERCENT UPTAKE OF MERCURY BY CELLS
100
90
80
70
60
50
40
30
20
10
0
0
24
72
120
168
240
288
HOURS AFTER ADDITION OF MERCURY
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OPTICAL DENSITY
AT 700 mμ

about one-fourth of the mercury added was immediately sorbed by both the live and the killed cells. After 24 hours, an unusually high uptake of mercury was noted in both cultures, with the live culture sorbing 77 percent, and the killed 67 percent. Because this experiment was, in effect, a replicate of the previous culture, the results can be compared.

In the short term experiment, Chlorella pyrenoidosa cells sorbed only about 30 percent after 24 hours with a maximum average uptake of 40 percent after 107 hours. Results showing twice this uptake in the long term study are not due to experimental error because mercury uptake remains fairly constant and high throughout the experiment period. No significant difference is shown in Table 7 between the 24 hour live uptake measurement and subsequent analyses. Although percent uptake by the killed cells declined substantially after the 24 hour sample, they maintained an average uptake of 50 percent throughout the experiment with no significant differences after the 72 hour reading. All the sorption sites may be filled within 72 hours.

The results of the long term mercury uptake experiment with Chlorella pyrenoidosa conflict with those of the short term experiment where uptake was relatively low even though the initial culture density was matched and both cultures were incubated and analyzed in the same manner. Therefore, the physical status, exact age, and amount of fresh media added may be critical conditions dictating the rate of mercury sorption.

Though uptake by the live cells was significantly greater than uptake by the killed cells over the 288 hour period, mercury uptake by live as well as

Table 7. Comparison of subsequent mercury uptake by Chlorella pyrenoidosa (long term experiment). T-tests comparing the mean percent mercury uptake of all subsequent samples of the live and the killed cultures of Chlorella pyrenoidosa through 288 hours with the mean percent mercury uptake by the live cells at 24 hours and by the killed cells at the 72 hour sampling.

Culture type	Comparison	t value
Live	$\bar{X}_{72}$ hrs. vs. $\bar{X}_{24}$ hrs.	1.105*
Live	$\bar{X}_{120}$ hrs. vs. $\bar{X}_{24}$ hrs.	0.822*
Live	$\bar{X}_{168}$ hrs. vs. $\bar{X}_{24}$ hrs.	2.621*
Live	$\bar{X}_{240}$ hrs. vs. $\bar{X}_{24}$ hrs.	0.331*
Live	$\bar{X}_{288}$ hrs. vs. $\bar{X}_{24}$ hrs.	1.095*
Killed	$\bar{X}_{120}$ hrs. vs. $\bar{X}_{72}$ hrs.	0.622*
Killed	$\bar{X}_{168}$ hrs. vs. $\bar{X}_{72}$ hrs.	0.675*
Killed	$\bar{X}_{240}$ hrs. vs. $\bar{X}_{72}$ hrs.	0.330*
Killed	$\bar{X}_{288}$ hrs. vs. $\bar{X}_{72}$ hrs.	1.720*

Degrees of freedom: 4
Tabular t value at the 0.05 level: 2.776

*Not significant at the 0.05 level, $P > 0.05$.

killed cells is virtually constant. Optical density increased rapidly in the live cultures, but of course remained constant for the killed. Because mercury sorption remained constant in the live as well as the killed cultures, the mechanism of mercury uptake appears to be predominantly passive.

The rate of mercury uptake expressed as mean percent uptake per optical density unit in Table 8 shows that the rate of uptake increased substantially only as culture density rose slightly during the first 24 hours incubation. Like the rate of uptake in the short term experiment (Table 6), a decline in the rate of uptake was noted after the culture entered the exponential growth phase, and this decline continued throughout the experiment.

Table 8. Rate of mercury uptake by Chlorella pyrenoidosa (long term experiment). Rate of mercury uptake in 288 hour experiment by Chlorella pyrenoidosa expressed as the mean percent uptake of three replicates per optical density unit for live culture.

Time in hours after mercury addition	Mean percent uptake	Optical density	Rate of uptake (percent per O.D. unit)
0	27	0.050	540
24	77	0.055	1400
72	69	0.135	510
120	78	0.300	260
168	85	0.340	250
240	77	0.450	171
288	69	0.725	95

Discussion of uptake analyses. Uptake experiments on Selenastrum capricornutum and Chlamydomonas reinhardi were of such short duration that no index of culture growth was obtained, and no rate of uptake could therefore be demonstrated. The passive mechanism of uptake was established by the statistical equivalence of mercury uptake by the live and the killed cultures in these experiments.

Results show that mercury uptake by live cells, presumably by a surface sorption process, is rapid, with about 25 percent of available mercury immediately sorbed and up to 80 percent sorbed after 24 hours in 0.1 ppm mercury. A control experiment also revealed that from 10-25 percent of the mercury in a culture containing 20 ppm mercury was sorbed in one-half hour.

The mercury from the medium was never totally depleted in the analyses made. One hundred percent uptake might have been expected in cultures

incubated for long periods of time because as cell number increases exponentially, the number of active sites for mercury sorption also increases. As in tolerance analyses, chemical binding of mercury to the medium probably reached an equilibrium with the binding capacity of the algae, preventing further mercury depletion.

Recommendations for further research

The hypothesis of chemical binding and the equilibrium of mercury between the algae and the medium could be investigated by employing a chemostat. In such experiments chelating agents could be added to mercury-containing algal cultures. A chemostat would enable continuous control and monitoring of the algal density and chemicals in the medium to determine whether constituents in the medium might limit mercury uptake. If a substance could effectively compete for mercury with organisms in natural systems and the competing substance was not detrimental to the environment, addition to an aquatic system might reduce mercury availability.

The magnitude and rate of mercury uptake differed from genus to genus. This variation among three members of the Chlorophyta prevents any blanket conclusions about mercury uptake by all algae. Phylogeny and the underlying morphology and nutritional characteristics could possibly take mercury uptake differ greatly between different forms.

Additionally, algae having a wide salt tolerance could be analyzed for mercury sorption in different saline concentrations. Algae native to marine, estuarine, and brackish waters are most vulnerable to mercury which becomes

more available as salt concentrations increase (Feick et al., 1972). These algae could be studied in media with different salt concentrations to determine tolerance levels and mercury uptake rates.

After the initial uptake of mercury in all cultures, the extent of mercury sorption remained constant throughout exponential growth. Samples of cultures in the phases of declining growth were not taken. Mercury content of the live and the killed cells never declined over the experiment period. However, the unialgal cultures were in inorganic media which restricted bacterial growth, and even formalin-killed cells appeared to retain their structural integrity throughout the experiments as verified by microscopy. The mercury uptake by mixed algal populations and by heavily bacterized algal cultures, particularly through the stationary and death phases, should be studied to determine whether live cells retain the mercury initially sorbed. If mercury is released through autolysis or bacterial degradation, the final sink for mercury should be established. The dynamics, chemistry, and mechanisms of mercury passage from the algae or the medium to bacteria and higher trophic levels should also be studied in greater detail.

The sorption of mercury by bottom sediments and the release and subsequent availability of mercury to algae is of importance ecologically, for if a high percentage of the mercury present could be effectively removed by a dense algal population, harvesting and disposal of the algae should be investigated as a possible means of significantly reducing mercury contamination in some aquatic systems.

Such proposed research was beyond the scope of this investigation but could lead to focal points of future studies. In 1967 the U.S. Public Health Service concluded:

> From our review of mercury as an environmental chemical contaminant it is obvious that a considerable amount of mercury has been cycled through our environment. . . . We have little or no information as to where the mercury that is being cycled through our environment is going. (Council on Environmental Quality, 1971, p. 12)

This investigation has demonstrated that algae can act as a sink for small quantities of mercury and may, at least temporarily, withdraw a significant percentage of available mercury present in a highly restricted, well controlled aquatic system.

SUMMARY AND CONCLUSIONS

1. An objective of this investigation was to study the inhibition of algal growth by low levels of mercury using two common species of freshwater algae, Chlorella pyrenoidosa and Selenastrum capricornutum.
2. Algistatic concentrations of mercury ranged from 1.0 to 2.0 ppm in Chlorella pyrenoidosa and 3.0 ppm proved lethal.
3. The inhibitory effect of mercury on the algae studied was dependent on culture density and the physical condition and age of the culture.
4. Algae studied seemed more resistant to mercury inhibition in protein-rich media, suggesting binding of mercury to the protein enabling greater tolerance.
5. In cultures with 1 drop inoculum and in sterile controls, 90 percent of added mercury was sorbed by the glass within 92 hours. To minimize glass sorption, mercury was added to dense algal cultures, and the cultures were maintained for only short periods of time.
6. Dense algal cultures were found to compete favorably with the glass for mercury.
7. A workable procedure was obtained for analyzing mercury in concentrations as low as 100 ppb in algae.
8. The percentage of available mercury sorbed by the cells at the time of sampling was found to be a reliable index of mercury uptake.

9. Another objective of this investigation was to study the rate, magnitude, and mechanism of mercury uptake by using initially dense unialgal live and killed cultures of Selenastrum capricornutum, Chlorella pyrenoidosa, and Chlamydomonas reinhardi.

10. The rate and extent of mercury uptake varies greatly from genus to genus as well as with the age and physical condition of the culture.

11. The mechanism of mercury uptake by the algae studied seemed to be strictly passive.

12. Once mercury was initially sorbed by cells the percent of mercury sorbed remained constant throughout exponential growth.

13. The fact that mercury uptake is passive does not preclude the possibility that live or dead algal cells concentrate mercury and pass it to higher trophic levels.

14. Because formalin-killed cells usually sorbed mercury as readily as live cells, dead algal cells as well as bacteria and sediments are probably as efficient as live cells as sinks for available mercury.

15. Becuase the medium was never entirely depleted of mercury by the algae, chemical binding of mercury with the medium is indicated, limiting mercury uptake.

LITERATURE CITED

Anderson, B. G. 1948. The apparent threshholds of toxicity to Daphnia magna for chlorides of various metals added to Lake Erie water. Transactions of the American Fisheries Society 78:111.

Bache, C. A., W. H. Butenman, and D. J. Lisk. 1971. Residues of total mercury and methylmercuric salts in lake trout as a function of age. Science 172:951-952.

Coleman Instrument Division. 1970. Mas 50 Mercury Analyzer operation instructions. Coleman Instruments Division of Perkin Elmer Corporation, Maywood, Illinois.

Coleman Instrument Division. 1971. Recommended procedure for analysis of mercury in various materials. Coleman Instruments Division of Perkin Elmer Corporation, Maywood, Illinois.

Council on Environmental Quality. 1971. Toxic substances. U.S. Government Printing Office, Washington, D.C.

Feick, G., R. A. Horne, and D Yeaple. 1972. Release of mercury from contaminated freshwater sediments by the runoff of road deicing salt. Science 175:1142-1143.

Fimreite, N. 1970. Mercury contamination of Canadian fish and fish-eating birds. Water Pollution Control 42:21-26.

Glooschenko, W. A. 1969. Accumulation of ^{203}Hg by the marine diatom Chaetoceros costatum. Journal of Physcology 5:224-226.

Grant, N 1971. Mercury in man. Environment 13(4):3, 8.

Harris, R. C., D. B. White, and R. B. Macfarlane. 1970. Mercury compounds reduce photosynthesis by plankton. Science 170:736-737.

Hatch, W., and W. L. Ott. 1968. Determination of sub-microgram quantities of mercury by atomic absorption spectrophotometry. Analytical Chemisty 40(14):2085-2087.

Horwitz, I. 1957. Observations of the effects of metallic mercury upon some microorganisms. Annals of the New York Academy of Sciences 65:430, 437-439.

Imura, N., E. Sukgawa, S. Pan, K. Nagao, J. K. Kim, T. Kwan, and T. Ukita. 1971. Chemical methylation of inorganic mercury with methylcobalamin, a vitamin B_{12} analog. Science 172:1248-1249.

Jensen, S., and A. Jernelov. 1969. Biological methylation of mercury in aquatic organisms. Nature 233:753-754.

Jernelov, A. 1968. The menace of mercury. New Scientist 40:627.

Matson, R. S., B. E. Mustoe, and S. B. Chang. 1972. Mercury inhibition on lipid biosynthesis in freshwater algae. Environmental Science and Technology 6(2):158.

Matsumura, F., Y. Gotoh, and G. M. Boush. 1971. Phenylmercuric acetate: Metabolic conversion by microorganisms. Science 175:49-51.

Miller, G. E., P. M. Grant, R. Kishoe, J. Steinkruger, F. S. Rowland, and V. P. Guinn. 1972. Mercury concentrations in museum specimens of tuna and swordfish. Science 175:1121-1122.

Myers, J. 1962. Laboratory cultures, pp. 603-613. In R. A. Lewin (Ed.). Physiology and biochemistry of algae. Academic Press, New York.

Nelson, N. (Chairman of study group on mercury hazards.) 1971. Hazards of mercury. Environmental Research 4(1):24-26.

Provasoli, L., and I. J. Pintner. 1959. Artificial media for freshwater algae; problems and suggestions, pp. 84-96. In C. A. Tyron, Jr., and R. T. Hartman (Eds.). The ecology of algae. Special Publication Number 2. Pymatuning Laboratory of Field Biology, University of Pittsburg, Pittsburg, Pennsylvania.

Starr, R. C. 1964. The culture collection of algae at Indiana University. American Journal of Botany 51:1037-1038.

U.S. Geological Survey. 1970. Mercury in the environment. Geolgoical Survey Professional Paper 713. U.S. Government Printing Office, Washington, D.C.

Wallace, R. A., W. Fulkerson, W. D. Shulz, and W. S. Lyon. 1971. Mercury in the environment: The human element. Oak Ridge National Laboratory, Oak Ridge, Tennessee.

APPENDIX

Bristol's Medium (Starr, 1964)

For each 1000 ml of medium required place the following amounts of stock solutions in 938 ml of Pyrex-distilled water.

# of ml	Stock solution	
10.00	$NaNO_3$	10.0 g/400 ml
10.00	$CaCl_2$	1.0 g/400 ml
10.00	$MgSO_4 \cdot 7H_2O$	3.0 g/400 ml
10.00	K_2HPO_4	3.0 g/400 ml
10.00	KH_2PO_4	7.0 g/400 ml
0.05	$FeCl_3$	1.0 g/100 ml
2.00	P IV metal solution	see below

Solidify with 15 g of agar per liter, if desired.

P IV Trace Metal Mix (Provasoli and Pintner, 1959)

To 500 ml of glass distilled water and the following amounts of chelating agent and metal salts:

$FeCl_3 \cdot 6\ H_2O$	0.097 g
$MnCl_2 \cdot 4\ H_2O$	0.041 g
$ZnCl_2$	0.005 g
$CoCl_2 \cdot 6\ H_2O$	0.002 g
Na_2MoO_4	0.004 g
Na_2EDTA	0.750 g

Proteose Medium (Starr, 1964)

For each 1000 ml of medium required add 1.0 g proteose peptone to 1000 ml of Bristol's medium (see above). Solidify with 15 g of agar per liter, if desired.

VITA

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